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Identification of Factor VIII Gene Mutations in Hemophilia A Patients from Punjab

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Abstract: The main objective of this preliminary study was to find out the pattern of mutations in the Factor VIII gene that cause hemophilia A in Pakistani population and compare the result of mutational analysis with other ethnic groups in term of genetic defects and clinical manifestations of the disease. For factor VIII gene mutations, we initially focused on six coding regions (exons 4, 8, 11, 18, 23 and 24). The reason for studying these exons was their smaller sizes and high mutational rates. The DNA from 22 unrelated hemophilia A patients was analyzed and the results of the analysis showed that out of 22 patients, one patient with mild disease had a previously reported mutation in exon 23 at codon 2159, CGC ➔ CAC (arginine ➔ histadine). In other 3 unrelated patients, a single nucleotide change (TTC ➔ TTT) of codon 205 at position 615 in exon 4 was detected. This is an unreported silent mutation as both the codons TTC and TTT code for phenylalanine and has no effect on the structure and function of the protein. We could not detect any mutation in the remaining 18 patients in either of four exons 8, 11, 18 and 24 which suggest that mutations in these coding regions of factor VIII are uncommon in hemophiliac patients from Punjab. Our data indicate ethnic diversity when compared with other world populations and therefore calls for a detailed study into the molecular basis of classical hemophilia in Pakistan on larger group of patients.

Key words: Hemophilia · molecular genetics · Pakistan

INTRODUCTION

Hemophilia A (also known as classical hemophilia), is an X-linked recessive disorder of coagulation caused by mutation in the F8c gene. The mutation of F8c gene causes deficiency or dysfunction of clotting factor VIII, which is as an essential cofactor in cleavage of factor X by factor IXa in the presence of Ca2+ and phospholipids [1,2]. Hemophilia A is a pan ethnic disorder without racial predilection and has an estimated incidence of 1 in 5000-1000 in newborn males. The factor VIII gene has been mapped to the telomere end of the long arm of X chromosome at Xq28; it spans over 168 kb and consists of 26 exons which encode a polypeptide chain of 2351 amino acids. It is well documented that genetic defects that result in hemophilia are quite heterogeneous and are due to wide spectrum of mutations in FVIII gene. At present more than 950 different mutations in FVIII gene that produce variable degree of clinical manifestations in hemophilic patients have been documented from all over the world [3]. The spectrum of clinical severity approximately correlates with the assayed level of coagulation factor VIII in the plasma. i.e. severely affected individuals have <0.01 IU/dl (<1% of normal), while moderate have 0.01-0.05 iu /dl (1%-5% of normal); and mild have 0.05- < 0.40 iu /dl (>5% - <40% of normal) of coagulation factor in blood. In hemophilic patients, the abnormality of blood coagulation disorders manifest itself in early childhood and spontaneous and traumatic bleeds continue throughout the life. The main sites of bleeding are into joints and muscles, subcutaneous bruising and hemorrhage after dental extraction and surgery [4]. In Pakistan hemophilia A is one of the most commonly encountered hereditary bleeding disorder and many children present with mild to severe clinical manifestations in their early childhood. The true incidence of the disease however is unknown but it is probably high due to higher rate of consanguinity (45%). The molecular studies on hemophilia in Pakistan are completely lacking and thus

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need an investigation. The main objectives of this preliminary study are:

- To investigate the molecular basis of hemophilia A in population from Punjab.
- To compare the results of mutational analysis with other ethnic groups in term of genetic defect s and clinical manifestations of the disease.

MATERIALS AND METHODS

Blood samples from 22 hemophilia A patients (ages 4-25 years) were collected in a 5 ml EDTA tube from Lahore, Multan and Islamabad. These samples were processed immediately or frozen for long term preservation. Genomic DNA was extracted either from fresh or frozen blood using non-organic method [5]. The quantity of the extracted DNA solution was estimated by spectrophotometery and yield gel electrophoresis in TE solution. To synthesize primers suitable for the analysis of the selected regions, various primer choices were examined using the computer program Primer 3, MELT 87 etc. A total of 3 sets of primers for each exon were chosen. The primers were mostly 25 nucleotide G+C rich sequence. Sizes of the amplified products ranged from 225 to 374 bp. 12 primers flanking, two for each of 6 exons 4, 8, 11, 18, 23, 24 were designed in house and were synthesized by solid-phase phosphotriester technique. These primers were then utilized to amplify selected portions of DNA using PCR amplification technique [6]. Amplification was carried out in a BIO-RAD I-Cycler and 1-2 µL of genomic DNA was used as template. Quality and quantity of amplification was checked on an agarose gel using ethidium bromide staining. All fragments exhibiting aberrant banding pattern were then subjected to cycle sequencing using Big Dye terminator technology. Sequencing of the amplified DNA products was done by dideoxy termination method on ABI 3100 genetic analyzer capillary electrophoresis system (Applied Biosystems).

RESULTS

DNA analysis were performed on 22 unrelated hemophilia A patients and Table 1 summarizes the clinical manifestations and results of mutational analysis of exons 4, 8, 11, 18, 23 and 24 of the Factor VIII gene.

The two mutation described in the exons 4 and 23 of FVIII gene were reconfirmed on repeat samples and were searched on www.HAMSTeRS data base. The mutation CGC ➔ CAC (Arg ➔ His) in exon 23 was a known mutation whereas TTC ➔ TTT (Phe ➔ Phe) in exon 4 was an unreported silent mutation.

Table 1: Results of the mutational analysis in 22 Hemophilia A patients

Patient #	Patient Name	age	Clinical Manifestation	DNA Mutation	Remarks.
P1	Fatima	21	knee joint swelling	TTC ➔ TTT	silent mutation (Phe ➔ Phe)
P2	Ahmed	6	gum bleeding.	nil	
P3	Zafar	14	gum bleeding, swelling	nil	
P4	Madassar	12	swollen ankle, gum bleeding	nil	
P5	Saleem	24	knee joint bleeding.	nil	
P6	Faisal	25	nasal and gum bleeding	nil	
P7	Javed	16	knee joint swelling	nil	
P8	Qadeer	9	nasal bleeding	nil	
P9	Muneeb	17	gum bleeding	nil	
P10	Rasheed	15	nasal bleeding; gum swelling	TTC ➔ TTT	silent mutation (Phe ➔ Phe)
P11	Uzair	4	gum bleeding and swelling	nil	
P12	Talha	23	knee joint swelling; gum bleeding	CGC ➔ CAC	Arg ➔ His
P13	Subhan	10	gum bleeding and swelling	nil	
P14	Afzal	17	nasal bleeding	nil	
P15	Ali Raza	7	gum bleeding	nil	
P16	Daniyal	15	ankle joint swelling	TTC ➔ TTT	silent mutation (Phe ➔ Phe)
P17	Fayyaz	24	knee joint swelling	nil	
P18	Babar	13	nasal bleeding	nil	
P19	Nabeel	9	gum bleeding	nil	
P20	Zubair	11	gum swelling and bleeding	nil	
P21	Dawood	27	knee joint swelling	nil	
P22	Asghar	15	swelling of ankle joint.	nil	

DISCUSSION

In this study we analyzed the DNA from 22 hemophilia A patients (aged 4-25 years) with mild to severe form of disease. All these patients had normal prothrombin time (PT) of 13-15 sec; abnormal activated partial thromboplastin time (APTT) of 73-158 sec (normal 34 seconds) and factor VIII activity of 1-10% of the normal. The main complaints at the time of presentation in these patients were pain in all major joints, in bilateral knee and joint swelling, nose, gum and teeth bleeding.

The results of DNA analysis through sequencing and BLAST analysis showed that out of 22 hemophilia A patients, only one patient with severe

clinical manifestation who was from Islamabad had a mutation in exon 23 at codon 2159, CGC ➔ CAC (Arginine ➔ Histidine). This is a previously reported mutation that has been described in three different ethnic groups with mild to moderate severity [7]. This study therefore corroborate our initial contention that although the mutational analysis of FVIII gene can help us in understanding the molecular basis of the disease but it can not predict the clinical course as evident from the fact that although the hemophilia patients from northern Punjab and other ethnic groups carried the same mutation CGC ➔ CAC (Arg ➔ His) but the clinical manifestation were different. The hemophilia patient from Punjab presented with severe form of disease but it was mild in patients from ethnic groups.

Arginine is involved in about 25% of all missense mutations reported in exon 4 and the substitution of arginine by another amino acid has been reported to cause mild to severe hemophilia. It also plays an important role in the stability and function of the protein. The importance of arginine for factor VIII activity has already been proven by directed mutagenesis at the activation (thrombin cleavage) and in activation sites [8].

We also investigated the other exons namely 4, 8, 11, 28 and 24 of FVIII gene. We were expected to find out some of previously identified and also some unknown mutations in these exons as at least 159 different types of mutations in these coding regions have been reported in hemophilia A patients from other parts of the world. We could detect only one mutation in exon 4 in three unrelated hemophilia A patients from Lahore, Multan and Islamabad with mild clinical presentation. This mutation was a single nucleotide change (TTC ➔ TTT) of codon 205 at position 615 and is a silent mutation as both codons TTC and TTT represent phenylalanine; however this mutation is a un reported one that has not been described in any other ethnic group. This nucleotide change (C ➔ T) at position 615 shows no effect on the structure and function of the protein. In the remaining 18 hemophilia A patients no mutation was detected in either exon 8, 11, 18 or 24 of FVIII gene indicating that the mutations in these coding regions are uncommon in Pakistani hemophilia patients but require further investigations. Our data therefore suggests that hemophilia A patients from Punjab are ethically diverged and the mutations that are present in other ethnic groups may be uncommon to Punjabi hemophilia A patients. Similar results were reported on the genetic analysis of hemophilia A from Bulgaria in which no mutation was detected in 34 patients in exon 11, 14, 24 of factor VIII gene [9]. This ethnic diversity therefore calls for a detailed study into the molecular basis of classical hemophilia in Pakistan as the information obtained from such study can not only be helpful in the understanding the molecular basis of the disease, but the data obtained from such study can be useful in developing a cost control treatment of hemophilic patients in Pakistan. It can also be useful for developing appropriate health care delivery system. Another reason that we might have missed the mutations in exons 8, 11, 18 and 24 of factor VIII gene is our hemophilia A patents is the sample size of investigation, which was small and therefore require DNA analysis on a large number of hemophilia A patients from Punjab to establish or rule out ethnic diversity and to obtain accurate data on molecular basis of the disease which is lacking currently.

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